



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

5 Takeshi Shigematsu et al Art Unit: 1641  
Serial No. 10/009,151 Examiner: Padmanabhan, Kartic  
Filed: April 16, 2002 Confirmation No. 8643  
Title: STABILIZED HUMAN DENATURED LIPOPROTEIN AND METHOD FOR  
PRODUCTION THEREOF

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DECLARATION UNDER 37 C.F.R. 1.132

HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS  
UNITED STATES PATENT AND TRADEMARK OFFICE  
15 WASHINGTON, D.C. 20231

Sir:

Being duly sworn, I, Hiroaki Kohno, a citizen of Japan,  
20 residing at c/o Kyowa Medex Research Laboratories, KYOWA  
MEDEX CO., LTD. 600- 1 , Aza-Kamiyamaji, Minamiishiki,  
Nagaizumi-cho, Sunto-gun, Shizuoka 411-0932 Japan, depose  
and say:

I. I am one of the co-inventors in the above-referenced  
25 application, and a Veterinary Doctor, a Doctor of Philosophy  
(Medical Science), and a Japan Certified Clinical Chemist  
(Japan Society of Clinical Chemistry) as well as a Vice Director  
of Fuji research Laboratory of KYOWA MEDEX CO., Ltd., and  
a Manager: combination of offices of HQ International Research  
30 and Development, an assignee of record of this application,  
on the subject matters relating to this application.

I graduated from Department of Veterinary, College of

Agriculture, University of Osaka Prefecture, Osaka, Japan in March 1980; from Department of Veterinary Physiology, College of Agriculture, University of Osaka Prefecture, Osaka, Japan in March 1982 and obtained a Master Degree; and also 5 from Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan in March 1986 and got a Ph.D.

From April, 1986 up till the present, I have been an employee of KYOWA MEDEX CO., Ltd. at Research and Development 10 Department of this company, I have been engaged in the research work with respect of glycolipid, especially modified lipoprotein.

I am well acquainted with all the co-inventors and experimental data in this case, having worked with them on 15 the development thereof, in this application, I am expressing their opinion, as well as my own.

II. In order to investigate the nature of the denatured lipoprotein produced by a freezing and melting step, I made 20 the experiments as follow:

<Experiment>

1. Preparation of LDL (Sample 1)

LDL fraction was obtained by the same procedure of Example 25 1(1). The concentration of LDL protein was adjusted to 1.4 mg/mL.

2. Preparation of Denatured LDL

2-1. Preparation of Denatured LDL by a freezing and melting 30 step of this invention (Sample 2)

The LDL obtained in 1. above was frozen at -40 °C and then melted by being stood at room temperature. The desired

denatured LDL was obtained by repeating the above steps three times.

#### 2-2. Preparation of Copper-oxidized LDL (Sample 3)

To 0.3 mL of the LDL solution obtained in 1. above, 10  $\mu$ L of an aqueous copper sulfate solution (0.5 mmol/L) was added dropwise. The resultant mixture was reacted at 37°C for 6 hours. Then, the reaction solution was dialyzed against an aqueous NaCl solution (0.15 mol/L) containing 1 mmol/L EDTA, to obtain a copper-oxidized LDL as the denatured LDL.

#### 2-3. Preparation of Acetylated LDL (Sample 4)

To 0.3 mL of the LDL solution obtained in 1. above, 0.3 mL of a saturated aqueous solution of sodium acetate was added and 4  $\mu$ L of acetic anhydride was further added dropwise, with stirred gently with a stirrer on ice. After the resultant mixture was continued to stand on ice for additional one hour, it was dialyzed against an aqueous NaCl solution (0.15 mol/L) containing 1 mmol/L EDTA, to obtain an acetylated LDL as the denatured LDL.

#### 2-4. Preparation of Saccharified LDL (Sample 5)

To 0.3 mL of the LDL solution obtained in 1. above, D-glucose was added so as to give the final concentration thereof to 400 mmol/L. The resultant mixture, after being reacted at 37°C for 3 days, was dialyzed against an aqueous NaCl solution (0.15 mol/L) containing 1 mmol/L EDTA, to obtain a saccharified LDL as the denatured LDL.

#### 2-5. Preparation of LDL treated with 4-hydroxy nonenal (4-HNE) (Sample 6)

To 0.3 mL of the LDL solution obtained in 1. above, 300

nmol of 4-hydroxy nonenal was added. The resultant mixture, after being reacted at 37°C for 3 days, was dialyzed against an aqueous NaCl solution (0.15 mol/L) containing 1 mmol/L EDTA, to obtain a LDL treated with 4-HNE as the denatured 5 LDL.

### 3. Analysis of Denatured LDL

#### 1) Analysis by agarose gel electrophoresis

The samples 1-6 obtained in 1. and 2-1. to 2-5. above 10 were subjected to electrophoresis using 0.8% agarose gel (produced by K.K. Helena Kenkyujyo, Japan under trademark "Coretoricombo CH"), to compare the mobilities. In this case, the voltage used was 400 V and the electrophoresis time was 40 minutes. After the electrophoresis, the bands were stained 15 using cholesterol and triglyceride in order to read the mobilities.

#### 2) Analysis by acrylamide gel electrophoresis

The samples 1-6 obtained in 1. and 2-1. to 2-5. above 20 were subjected to electrophoresis using 12% polyacrylamide gel containing Sudan Black B (Lipophor, produced by JOKOH CO., LTD, Japan), to compare the mobilities.

#### 3) Reactivity with DLH3 antibody

The samples 1-6 obtained in 1. and 2-1. to 2-5. above 25 were tested for reactivity with DLH3 antibody by ELISA method which was carried out in accordance with the same procedure as in Example 1(4) while using the samples 1-6 instead.

### 30 4. Results

For each the samples, the mobility and reactivity are shown in the following Table 1.

Table 1

Sample	Mobility		Reactivity
	Agarose gel	Acrylamide gel	
Sample 1 (Untreated)	174	169	No
Sample 2 (Invention)	173	170	Yes
Sample 3 (Oxidized)	140	167	Yes
Sample 4 (Acetylated)	48	180	No
Sample 5 (Saccharified)	166	102	No
Sample 6 (4-HNE)	167	133	No

## 5. Observation

It is noted from the results in the above Table that  
 5 the denatured LDL of this invention (Sample 2) has mobility  
 and reactivity with DLH3 antibody both different from the  
 other samples 1 and 3-6, indicating that the denatured LDL  
 produced by the freezing and melting step of this invention  
 is different from the prior denatured LDL's which are denatured  
 10 by the oxidation with copper, acetylation, saccharification,  
 and treatment with 4-HNE.

## III. CONCLUSION

As being clear from the Results and Observation of  
 15 EXPERIMENT, the denatured lipoprotein produced by a freezing  
 and melting step has properties significantly different from  
 conventional denatured lipoprotein such as copper-oxidized  
 lipoprotein, acetylated lipoprotein, saccharified  
 lipoprotein, and lipoprotein treated with 4-hydroxy nonenal.  
 20 Consequently, it is evident that the method for producing  
 denatured lipoprotein by a freezing and melting step is  
 distinct from the method disclosed in Yamada et al  
 (JP-A-9-297137) or Lang et al (EP-A-0 617 289).

I further declare that all the statements made herein  
of my own knowledge are true; and that all statements made  
on information and belief are believed to be true; and further  
that these statements were made with the knowledge that willful  
5 false statements and the like so made are punishable by fine  
or imprisonment, or both, under Section 1001 of Title 18 of  
the United States Code and that such willful false statements  
may jeopardized the validity of the application or any patent  
issuing thereon.

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Dated: 14th day of December, 2004

By Hiroaki Kohno

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Hiroaki Kohno